Ghrelin Neutralization by a Ribonucleic Acid-SPM Ameliorates Obesity in Diet-Induced Obese Mice

Lauren P. Shearman, Sheng-Ping Wang, Steffen Helmling, D. Sloan Stribling, Paul Mazur, Lan Ge, Liyang Wang, Sven Klussmann, D. Euan Macintyre, Andrew D. Howard, and Alison M. Strack

Departments of Pharmacology (L.P.S., S.-P.W., D.S.S., D.E.M., A.M.S.) and Metabolic Disorders (P.M., L.G., L.W., A.D.H.), Merck Research Laboratories, Rahway, New Jersey 07065; and Noxxon Pharma AG (S.H., S.K.), 10589 Berlin, Germany

Ghrelin, an acylated peptide secreted from the stomach, acts as a short-term signal of nutrient depletion. Ghrelin is an endogenous ligand for the GH secretagogue receptor 1a, a G protein-coupled receptor expressed in the hypothalamus and pituitary. We used a synthetic oligonucleotide, NOX-B11-2, capable of specific high-affinity binding to bioactive ghrelin to determine whether ghrelin neutralization would alter indices of energy balance *in vivo*. This novel type of ghrelin-blocking agent, called an RNA Spiegelmer (SPM), is a polyethylene glycol-modified L-RNA oligonucleotide, the nonnatural configuration of which confers *in vivo* stability. NOX-B11-2 blocked ghrelin mediated activation of GH secretagogue receptor 1a in cell culture (IC$_{50}$ ~5 nM). We explored the effects of acute NOX-B11-2 administration on ghrelin-induced feeding in mice. NOX-B11-2 (66 mg/kg, sc) blocked ghrelin-induced feeding and was without effect on feeding evoked by an orally active nonpeptide ghrelin receptor agonist. We demonstrated that selective ghrelin blockade effectively promoted weight loss in diet-induced obese (DIO) mice. Chronic infusion of NOX-B11-2 (33 mg/kg·d, sc) to DIO mice evoked body weight loss for 13 d and reduced food intake and fat mass relative to control SPM-infused mice. In a 7-d study, DIO mice infused with NOX-B11-2 (33 mg/kg·d, sc) showed body weight loss, compared with animals receiving control SPM. This effect was directly mediated by SPM neutralization of ghrelin because NOX-B11-2 administration to ghrelin-deficient mice resulted in no weight loss. The decreased obesity observed in SPM-treated DIO mice provides validation for ghrelin neutralization as a potential antiobesity therapy. (*Endocrinology* 147: 1517–1526, 2006)

G HRELIN IS AN acylated 28-amino acid peptide secreted primarily from the stomach, which acts as a short-term signal of nutrient depletion. Ghrelin is an endogenous ligand for the GH secretagogue receptor 1a (GHS-R1a), a G protein-coupled seven-transmembrane spanning receptor predominantly expressed in the hypothalamus and pituitary (1, 2). Ghrelin stimulates short-term food intake in rodents whether injected centrally or peripherally (3–5). In addition to its feeding effects, chronic administration of ghrelin durably increases body weight and adiposity in rodents via reduction in energy expenditure and alteration of fuel use(6). Ghrelin-deficient mice appear to be phenotypically normal with respect to their food intake and body weight gain (7); however, another group detected increased fat use and reduced fat mass (8). The major site of the orexigenic action of ghrelin is the hypothalamus. Ghrelin released by the stomach, or administered peripherally, activates GHS-R1a-expressing arcuate neurons at the base of the hypothalamus to release neuropeptide Y (NPY). Disruption of NPY and agouti-related protein (AgRP) using null mutant mice (9) or antibodies and antagonists (3) abolishes the orexigenic

effect of ghrelin, indicating that ghrelin action depends on functional NPY/AgRP neurons. Additionally, ghrelin signaling has been shown to depend on the integrity of the melanocortin system because melanocortin receptor-3 and -4 double-knockout mice have an attenuated response to ghrelin (9). Ghrelin has also been shown to interact with the orexin system to induce feeding (10).

In both rodents and humans, ghrelin levels rise preprandially and fall postprandially, suggesting that ghrelin serves a role in meal initiation and possibly satiation (11). Intravenous ghrelin increases sensations of hunger and food intake in humans (12). In several studies in patients undergoing gastric bypass surgery, a postsurgical reduction in plasma ghrelin was generally noted. Diet-induced weight loss is also associated with increased plasma ghrelin, supporting the notion that ghrelin is associated with long-term regulation of body weight (13). Patients with Prader-Willi syndrome have high circulating levels of ghrelin, which may drive their voracious appetite, hyperphagia, and obesity (14). Together, these data from rodent and human suggest a potential role for ghrelin in consummatory ingestive behavior and energy homeostasis.

We used a synthetic polyethylene glycol (PEG)-modified L-RNA oligonucleotide capable of specific high-affinity binding to acylated ghrelin to determine whether the hormone's neutralization alters indices of energy balance *in vivo*. This novel type of ghrelin-blocking agent, called SPM NOX-B11-2 (German *Spiegel* means mirror) consists of nonnatural L-ribose nucleotides, resulting in a mirror image configuration that renders the oligonucleotide resistant to nuclease digestion and therefore confers *in vivo* stability (15). NOX-B11-2

was designed to bind specifically and avidly to acylated (bioactive) ghrelin, thus preventing it from activating the GHS-R1a. It is the successor molecule of RNA-Spiegelmer (SPM) NOX-B11, which was previously shown to bind octanoylated ghrelin with low nanomolar affinity, requiring only the N-terminal 5 amino acids for the interaction (15). NOX-B11 inhibits ghrelin action in vitro and PEG-modified NOX-B11 was shown to inhibit ghrelin-mediated GH release and food intake in rats (15, 16).

We determined whether a second-generation antighrelin SPM, NOX-B11-2, blocked ghrelin mediated activation of GHS-R1a in cell culture. We first evaluated the effects of NOX-B11-2 on food intake induced by ghrelin or an orally active nonpeptide GHS-R1a agonist, Compound A, in mice. We next determined the effect of chronic peripheral infusion of NOX-B11-2 on indices of energy balance including body composition, food intake, and body weight gain in diet-induced obese (DIO) mice. In addition, we infused NOX-B11-2 peripherally in ghrelin-null ($Ghrl^{-/-}$) and wild-type mice to determine whether the observed effects of chronic NOX-B11-2 infusion were ghrelin mediated. Our results demonstrate that neutralization of acylated ghrelin suppresses ghrelin-induced food intake and causes mechanism-based body weight loss in mice.

Materials and Methods

Animals

All animal protocols used in these studies were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ). Mice were individually housed in microisolator cages on a 12-h light, 12-h dark cycle (lights on at 0500 h). Male C57BL/6N mice were purchased from Taconic Farms, Inc. (Germantown, NY). Wild-type ($Ghrl^{+/+}$) and $Ghrl^{-/-}$ mice littermate mice were generated by intercrossing $Ghrl^{+/-}$ mice, which had been backcrossed to C57BL/6N for three generations. All of the experimental mice were provided with ad libitum access to water and either regular mouse chow (Teklad 7012; 13.4% kcal from fat; 3.41 kcal/g; Harlan Teklad, Madison, WI) or high-fat diet (S3282; 59.4% kcal from fat, 5.29 kcal/g; Bioserv, Frenchtown NJ). DIO mice were fed a high-fat diet after weaning to induce obesity.

Compounds

Native human ghrelin (1–28 with Ser-3 octanoyl group) was synthesized by Mimotopes (Chiron, Emeryville, CA) or Synpep Corp. (Dublin, CA). Ghrelin peptidomimetic Compound A was prepared by Merck Research Laboratories (17). Ac-[Nle⁴, Asp⁵, D-Phe⁷, Lys¹⁰]a-MSH 4–10-NH₂ (MT-II) was purchased from Bachem/Peninsula Laboratories, Inc. (San Carlos, CA).

In vitro binding and functional assays

Peptides and nucleic acids. Ghrelin used for determination of dissociation constants and IC₅₀ in cell culture was obtained from Bachem (Heidelberg, Germany). Ghrelin used in the in vivo assays was from Mimotopes (Chiron) or Synpep.

SPM NOX-B11-2 has the sequence 5'-CCGGUGAGGCA-(PEG)₄₀-GUAAGACCGAAGGUAACCAUCCUACCGG-3'. It is a derivative of SPM NOX-B11 whose selection has been described previously (15). SPM NOX-B11-2 is seven nucleotides shorter and contains a PEG₄₀ replacing the bases AUAAAAC at positions 12–19 of NOX-B11. The control SPM has the sequence 5'-UAAGGAAACUCCGUCUGAUGCGGUAGCGC-UGUCCAGACCU-3' and has been described previously. All SPMs (L-RNAs) were synthesized at NOXXON Pharma AG using standard phosphoramidite chemistry. L-Amidites were obtained from ChemGenes

Corp. (Wilmington, MA). All SPMs used in this study were modified with a 40-kDa PEG moiety as described previously (18).

Inhibition of ghrelin-R1a activation in cell culture. Stably transfected Chinese hamster ovary (CHO) cells expressing human GHS-R1a (Euroscreen, Gosselies, Belgium) were seeded with 5–7 × 10³ cells/well in a 96-well plate (Greiner, Frickenhausen, Germany) and grown overnight at 37 C with 5% CO₂ in UltraCHO medium (Cambrex, Verviers, Belgium) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml geneticin, and 2.5 µg/ml Fungizone. Various concentrations of SPM were incubated for 15–60 min with bioactive human ghrelin (5 nM) in ultra-CHO medium containing 5 mM probenecid and 20 mM HEPES (CHO-U+) at 24 C in a 0.2 ml low-profile 96-well plate. Cells were washed once with 200 µl of CHO-U+, loaded with pluronic 127, 50 µl of 10 µM fluo-4 indicator dye solution (Molecular Probes, Eugene, OR) in CHO-U+, and incubated for 60 min at 37 C. Cells were then washed three times with 180 µl of CHO-U+. Ninety microliters of CHO-U+ were added per well, and the cells were incubated with 10 µl of the preincubated SPM-ghrelin mixture. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm in a Fluostar Optima multidetection plate reader (BMG, Offenburg, Germany). The resulting Ca²⁺-associated fluorescence was measured. For each well, the difference between the maximum fluorescence and the baseline value was determined and plotted against ghrelin concentration or against concentration of SPM. The EC₅₀ or IC₅₀ values were read from the graphs.

Nuclear factor of activated T cells (NFAT) β-lactamase reporter assay. The NFAT β-lactamase assay is a calcium inducible G protein-coupled receptor reporter assay (19). Ghrelin-R stably transfected (GHS-R1a in pcDNA3 or pChreo) NFAT-Bla-CHO-K1 cells (Invitrogen, Carlsbad, CA) were constructed for the β-lactamase assay and were maintained in Iscove's DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.25 mg/ml G418, and 100 µg/ml Zeocin. Cells were plated at 6000 cells/well in clear-bottom black wall 96-well plates (Costar, Cambridge, MA) 48 h before the assay. Human acyl-ghrelin and SPMs were combined in complete cell media at final concentrations noted, preincubated at room temperature for 10 min, and added to the cells. The cells were incubated for 3 h at 37 C, 5% CO₂ aspirated, and loaded with CCF4-AM (esterified form of CCF4, coumarin cephalosporin fluorescein) substrate (Invitrogen) for 1 h at room temperature, according to Invitrogen GeneBLAzer protocols. The background subtracted fluorescence emission ratio (460/530 nm) was obtained on a Analyst-HT (Molecular Devices, Sunnyvale, CA). Dose-response curves were plotted using Prism software (GraphPad, San Diego, CA).

Ghrelin-stimulated food intake

We used a daytime paradigm, a period when mice refrain from eating and endogenous ghrelin levels are at their nadir. Chow-fed male C57BL/6N mice (n = 8/group) received vehicle (PBS), NOX-B11-2, or control SPM (both at 30 nmol/mouse; ~66 mg/kg, sc) 3 h before saline or ghrelin challenge (20 µg/mouse; 0.6 mg/kg, ip) during the early light phase (0900 h, lights on at 0500 h). Food intake was measured at T0, 0.5, 1, 1.5, 2, 3, and 4 h after vehicle or ghrelin treatment. All food intake values were reported as mean ± SEM and analyzed by two-tailed, unpaired Student's t test. P < 0.05 was reported as significant.

Ghrelin receptor agonist-stimulated food intake

Chow-fed C57BL/6 male mice (n = 8/group) received vehicle (PBS), NOX-B11-2 or control SPM (both at 30 nmol/mouse; approximately 66 mg/kg, sc) 3 h before vehicle (10% Tween 80 in 0.25% methylcellulose, orally) or Compound A challenge (20 mg/kg, orally) during the early light phase (0900 h, lights on at 0500 h). Other positive control groups included mice dosed ip with ghrelin (0.6 mg/kg) ± NOX-B11-2 or inactive control SPM. Food intake was measured at T0, 0.5, 1, 1.5, 2, 3, and 4 h after vehicle, Compound A, or ghrelin treatment. All food intake values were reported as mean ± SEM and analyzed by two-tailed, unpaired Student's t test. P < 0.05 was reported as significant.

Chronic infusion study with antighrelin SPM NOX-B11-2 in DIO mice

Male DIO C57BL/6N mice were individually housed in a room with a 12-h light photoperiod. Mice were allowed *ad libitum* access to water and Bioserv high-fat diet (S3282). Mice (n = 8–10/group) were fed high-fat diet from arrival and for the duration of the study (~5 months on high-fat diet; 47 g average body weight). Baseline body composition of the mice was determined by Minispec nuclear magnetic resonance (NMR) analysis on d −7. On d 0, osmotic minipumps (Durect Corp., Cupertino, CA) were implanted sc under isoflurane anesthesia. NOX-B11-2, control SPM (both at 120 nmol/kg, 15 nmol/d; 1.6 mg/d; 33 mg/kg/d) or vehicle (PBS) was infused sc through an implantable infusion pumps (Alzet model 2002, 0.5 μl/h). The melanocortin receptor pan-agonist MT-II (0.5 mg/d; 10 mg/kg/d) was infused as a positive control for food intake and body weight suppression. Food intake and body weight change were monitored daily. On d 13, rectal temperature was measured at 0800 h (3 h after lights on) followed by NMR analysis at 1500 h. On d 14, blood samples were collected via cardiac puncture for leptin, insulin, ghrelin, and IGF-I analysis. Fat pads (epididymal, retroperitoneal, mesenteric) were weighed. Serum was analyzed for glucose, triglyceride, and total cholesterol levels.

Chronic infusion study with antighrelin SPM NOX-B11-2 in DIO ghrelin deficient and wild-type mice

All wild-type and ghrelin-deficient mice used in the infusion studies were fed high-fat diet and rendered DIO. A 7-d infusion study was performed in *ghrelin*-null and wild-type mice rendered DIO. All mice (n = 7–8 per group) were maintained on a high-fat diet at Taconic for 4 wk before arrival and for the duration of the study [3 months on high-fat diet (Bioserv; S3282); 41 g average body weight; 32% fat for both genotypes]. On d −7, baseline body composition was determined by Minispec NMR analysis. NOX-B11-2 or control SPMs (120 nmol/kg; 15 nmol/d; 1.6 mg/d; 33 mg/kg/d; in PBS) were infused sc through implantable infusion pumps (Alzet model 2002, 0.5 μl/h). MT-II (0.5 mg/d; 10 mg/kg/d) was infused as a control for food intake and body weight suppression. Food intake and body weight change were monitored daily. NMR analysis was performed on d 7.

RIAs and ELISAs

Plasma levels of leptin were assayed using a mouse leptin ELISA kit (catalog no. 90030) from Crystal Chem Inc. (Downers Grove, IL) using the manufacturer's instructions. Plasma levels of insulin were assayed using an ultrasensitive rat insulin ELISA kit (catalog no. 90060) and mouse insulin standards from Crystal Chem, according to the manufacturer's instructions. Serum IGF-I levels were measured using a mouse/rat IGF-I RIA kit (catalog no. DSL-2900) from Diagnostic Systems Laboratories, Inc. (Webster, TX) according to the manufacturer's instructions.

Acyl ghrelin levels in plasma were assayed using an active ghrelin ELISA kit (catalog no. EZGAC-86K, Linco Research, Inc., St. Charles, MO). Sample preparation was modified according to the following steps: 1) blood was drawn into prechilled EDTA-coated tubes and mixed gently by inversion; 2) 250 μl blood were added a chilled Eppendorf tube containing 2.5 μl of 2 M Pefabloc (AEBSF, Roche Diagnostics, Indianapolis, IN); 3) samples were centrifuged at 10,000 rpm for 10 min (4 C); and 4) 60 μl of plasma were added to a 96-well plate with 6 μl of 1 N HCl. Samples were stored at −80 C until assayed. Samples were diluted 1:2.5 with assay buffer, and the manufacturer's instructions were followed.

Total ghrelin was assayed in EDTA-plasma using a ghrelin RIA kit (catalog no. RK-031-31) from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Samples were diluted 1:10 with RIA buffer and the assay was performed according to the manufacturer's instructions. Glucose, triglyceride, and total cholesterol levels were measured in serum using a Roche Hitachi 911 automated clinical chemistry analyzer.

Statistical analysis

The means ± SEM were determined by one-way ANOVA, followed by two-tailed, unpaired Student's *t* test. Differences were considered to be significant when $P < 0.05$.

Results

NOX-B11-2 binds and inactivates ghrelin

We demonstrated previously that SPM NOX-B11, a 47 nucleotide long L-RNA binds octanoyl ghrelin and suppresses ghrelin-induced GH release and food intake (15, 16). In the work presented here, we focused on aspects of ghrelin relating to energy metabolism such as food intake, body weight, and body composition. To address these questions we worked with ghrelin-binding SPM NOX-B11-2, the successor molecule of NOX-B11. SPM NOX-B11-2 binds ghrelin with the same affinity as NOX-B11 and has the same specificity for octanoyl ghrelin (data not shown). NOX-B11-2 blocks ghrelin-mediated activation of GHS-R1a in cell culture (fluorimetric imaging plate reader calcium mobilization assay) with an IC_{50} of approximately 7 nM (Fig. 1). In a complementary β-lactamase assay, NOX-B11-2 inhibited ghrelin activation of mouse and human GHS-R1a with approximately 10-fold higher IC_{50} values between 69 and 136 nM (Fig. 2 and Table 1), perhaps due to protocol differences. Taken together these results confirm that NOX-B11-2 interferes with the ghrelin-GHS-R1a interaction.

NOX-B11-2 blocks ghrelin-stimulated food intake

Ghrelin (0.6 mg/kg, ip) increased food intake significantly from 0.5 to 2 h after injection, compared with the vehicle control group ($P < 0.05$ *vs.* vehicle) (Fig. 3A). Pretreatment with anti-ghrelin SPM NOX-B11-2 blocked the ghrelin-induced increase in food intake from 0.5 to 2 h ($P < 0.05$ *vs.* vehicle/ghrelin). Control SPM pretreatment did not block food intake stimulation induced by ghrelin challenge or alter *ad libitum* food intake. NOX-B-11-2 administration alone had no effect on daytime food intake. These data indicate that peripheral administration of the antighrelin SPM NOX-B11-2 is capable of inhibiting ghrelin-stimulated food intake in mice.
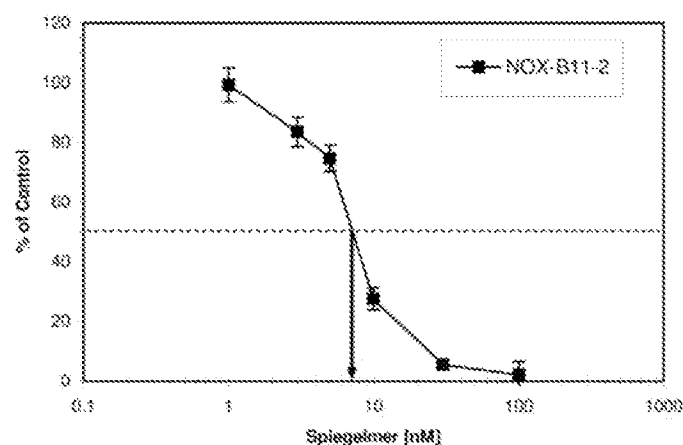

FIG. 1. SPM NOX-B11-2 inhibits ghrelin-mediated activation of GHS-R1a in cell culture. CHO cells expressing human GHS-R1a were stimulated with ghrelin at a concentration of 5 nM in the presence of various concentrations of SPM NOX-B11-2. The resulting Ca^{2+}-associated fluorescence was measured. The response to ghrelin binding to the receptor is suppressed by NOX-B11-2 in a dose-dependent manner.
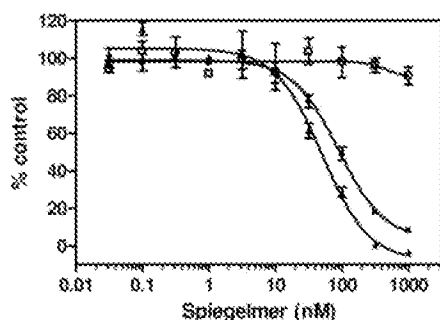
Fig. 2. Ghrelin mediated activation of the GHS-R1a is inhibited by SPM NOX-B11-2. Human GHS-R1a-expressing NFAT-Bla-CHO cells were stimulated with human ghrelin alone (control) or a preincubated mixture of ghrelin and SPM (□, 1 nM ghrelin + nonspecific L-RNA; ▲, 1 nM ghrelin + NOX-B11A; ●, 2 nM ghrelin + NOX-B11A) and assayed for β-lactamase activity. Data are mean ± SEM (duplicate) from a single experiment and are representative of four to six independent experiments.

NOX-B11-2 does not inhibit GHS-R1a agonist-stimulated food intake

GHS-R1a agonist Compound A (20 mg/kg, orally) increased food intake significantly from 0.5 to 4 h after dosing ($P < 0.05$ vs. vehicle) (Fig. 3B). Neither NOX-B11-2 nor the control SPM blocked food intake stimulation induced by Compound A treatment. In addition, NOX-B11-2 did not block food intake induced by a lower dose of Compound A (5 mg/kg, orally), which had equivalent food intake stimulation to ghrelin (data not shown). These data indicate that the antighrelin SPM NOX-B11-2 suppressed ghrelin-induced food intake but not food intake evoked by a nonpeptide GHS-R1a agonist.

Chronic infusion study with NOX-B11-2 in DIO mice

NOX-B11-2 infusion evoked weight loss, compared with controls (Fig. 4A). Significant body weight loss was observed with NOX-B11-2 infusion on d 1–10 and 12, compared with vehicle-treated mice, and d 1–13, compared with the control SPM-infused group ($P < 0.05$ vs. vehicle or control SPM). NOX-B11-2-infused mice gained 0.32 g of body weight, whereas those receiving control SPM gained 1.85 g by d 13. Control SPM infusion did not alter body weight gain relative to the vehicle control. Control SPM-infused mice gained an average of 1.85 g of body weight, whereas vehicle-infused mice gained an average of 0.91 g of body weight over the course of the study. Infusion of MT-II, a melanocortin receptor agonist serving as the positive control, evoked body weight loss when compared with vehicle treatment from d 1 onward (−1.62 g on d 13; $P < 0.05$ vs. vehicle).

NOX-B11-2 infusion decreased food intake significantly (Fig. 4B). Daily food intake was reduced significantly by

TABLE 1. NOX-B11-2 inhibition (IC$_{50}$) of human and mouse GHS-R1a in β-lactamase assay

h-Ghrelin (nM)	β-Lactamase IC$_{50}$ (nM; mean ± SD)	
	h-GHS-R1a	m-GHS-R1a
1	73 ± 34 (n = 6)	69 ± 21 (n = 4)
2	136 ± 48 (n = 6)	131 ± 59 (n = 4)

h, Human; m, mouse.

NOX-B11-2 infusion on d 1–4, compared with both vehicle and control SPM groups ($P < 0.05$ vs. vehicle or control SPM). MT-II infusion decreased daily food intake from d 1 to 6 ($P < 0.05$ vs. vehicle). Significant effects on cumulative food intake were observed on d 1–8, compared with the vehicle group, and d 1–13, compared with the control SPM group (39.33 vs. 42.61 g on d 13; $P < 0.05$ vs. control SPM) (Fig. 4C). Infusion of the control SPM did not alter cumulative or daily food intake. MT-II infusion reduced cumulative food intake significantly (35.94 vs. 40.17 g on d 13; $P < 0.05$ vs. vehicle).

We calculated feed efficiency (weight gain per kilocalorie ingested) from d 1–5 and 6–13 (Fig. 4D). This ratio indicates how efficiently the food energy was used for accretion of body mass. Feed efficiency was reduced by NOX-B11-2 infusion on d 1–5 ($P < 0.05$ vs. vehicle or control SPM), and this effect was not observed from d 6 to 13, suggesting that the transient reduction in weight gain was not simply due to reductions in food intake. In contrast, MT-II reduced feed efficiency from d 1 to 13 ($P < 0.05$ vs. vehicle), suggesting increased energy expenditure.

Treatment with NOX-B11-2 altered body composition of DIO mice (Fig. 4E). Fat mass content of NOX-B11-2-infused mice was decreased, compared with baseline (d −7) ($P = 0.05$ vs. vehicle; $P = 0.06$ vs. control SPM). Lean mass content was not affected by NOX-B11-2 infusion, compared with baseline (d −7). White adipose tissue depot weights were not altered by NOX-B11-2 infusion. Control SPM infusion did not alter body composition or white adipose tissue weights, compared with baseline or vehicle-treated animals. MT-II-infused mice gained less fat and lean mass over the 14-d infusion period ($P < 0.05$ vs. baseline and vehicle) and had significantly less retroperitoneal fat relative to vehicle infused controls (0.69 vs. 0.78 g; data not shown). We calculated fat mass corrected for body weight on d 13 (Fig. 4F). NOX-B11-2 infusion reduced fat mass to a small extent on d 13 relative to vehicle and control SPM groups ($P < 0.05$ vs. vehicle or control SPM). MT-II reduced fat mass on d 13 ($P < 0.05$ vs. vehicle). NOX-B11-2 infusion induced a small but significant increase in rectal temperature on d 13, compared with the control SPM group (36.78 ± 0.07 vs. 36.51 ± 0.07 C; $P < 0.05$ vs. control SPM). MT-II infusion did not alter rectal temperature relative to vehicle controls [36.39 ± 1.0 vs. 36.66 ± 0.06 C (vehicle)].

Acyl ghrelin levels were increased significantly by MT-II (29.4 pM; $P = 0.005$) and NOX-B11-2 infusion (170.8 pM) relative to the vehicle-treated controls (12.4 pM; $P < 0.0001$) (Fig. 4G). The magnitude of the increase in acyl ghrelin in NOX-B11-2-infused mice was greater than that observed with MT-II infusion. Acyl ghrelin levels were also increased significantly in the NOX-B11-2-treated mice relative to the control SPM infused mice (170.8 vs. 8.9 pM; $P < 0.0001$). NOX-B11-2 infusion reduced leptin and triglyceride levels relative to vehicle controls ($P = 0.02$ and $P = 0.05$ vs. vehicle, respectively). Leptin and triglyceride concentrations between control SPM and NOX-B11-2-infused mice were not different on d 13. There were no apparent changes in insulin, IGF-I, glucose, or total cholesterol levels. MT-II infusion reduced leptin and insulin levels significantly ($P = 0.018$ and $P = 0.026$ vs. vehicle, respectively). Data are shown in Table 2.

A

B

Fig. 3. SPM NOX-B11-2 inhibits orexigenic effects of peripheral ghrelin in mice. Chow-fed male C57BL/6N mice (n = 8/group) received NOX-B11-2 or control SPM (both at 30 nmol/mouse; ~66 mg/kg, sc) 3 h before ghrelin (20 μg/mouse; 0.6 mg/kg, ip) or Compound A (Cpd A; 20 mg/kg, orally) challenge during the early light phase (0900 h, lights on at 0500 h). A, NOX-B11-2 pretreatment (30 nmol/mouse, 66 mg/kg, sc) blocked ghrelin-stimulated food intake in mice. Ghrelin increased food intake from 0.5 to 2 h after injection (*P* < 0.05 *vs.* vehicle). Pretreatment with NOX-B11-2 blocked ghrelin-stimulated food intake from 0.5 to 2 h after ghrelin injection (*P* < 0.05 *vs.* vehicle/ghrelin). Neither NOX-B11-2 nor control SPM altered food intake when administered alone. Control SPM did not block food intake stimulation induced by ghrelin. B, NOX-B11-2 pretreatment (66 mg/kg, sc) did not alter food intake induced by GHS-R1a agonist Compound A (20 mg/kg, orally) in mice. Compound A increased food intake from 0.5 to 4 h after dosing (*P* < 0.05 *vs.* vehicle). Neither NOX-B11-2 nor the control SPM blocked food intake evoked by Compound A.
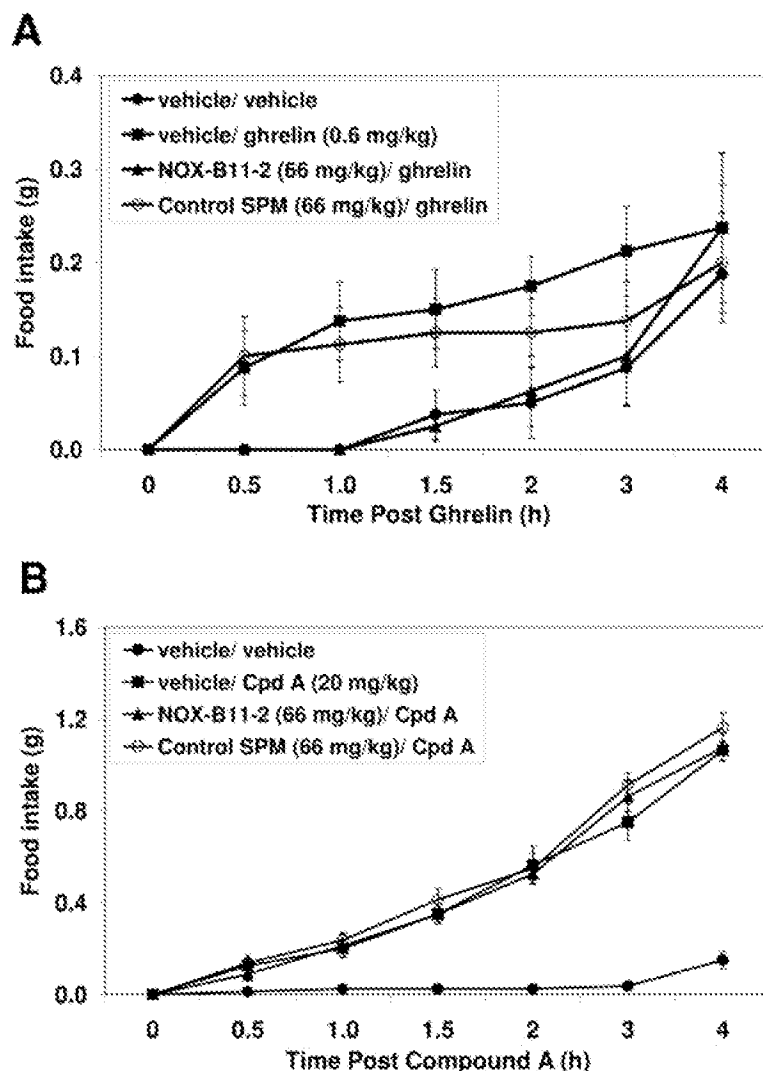
Chronic infusion study with NOX-B11-2 in DIO ghrelin-deficient and wild-type mice

In wild-type mice, significant body weight loss was observed with NOX-B11-2 infusion on d 1–6, compared with wild-type mice infused with the control SPM (*P* < 0.05 *vs.* control SPM) (Fig. 5A). NOX-B11-2 did not alter body weight in *Ghrl*−/− mice (Fig. 5B). The positive control, MT-II, reduced body weight gain significantly from d 1 onward in both wild-type and *Ghrl*−/− mice when compared with their respective control SPM groups (*P* < 0.05 *vs.* respective control SPM groups) (Fig. 5, A and B).

NOX-B11-2 infusion (33 mg/kg·d, sc) reduced daily food intake on d 1 in wild-type mice (Fig. 5C) (*P* < 0.05 *vs.* control SPM). NOX-B11-2 infusion did not alter food intake of *Ghrl*−/− mice (Fig. 5D). MT-II significantly suppressed daily food intake of wild-type mice from d 1 to 3 and *Ghrl*−/− mice from d 1 to 5 (*P* < 0.05 *vs.* respective control SPM groups). MT-II suppressed cumulative food intake of wild-type and *Ghrl*−/− mice from d 1 onward (*P* < 0.05 *vs.* control SPM).

NOX-B11-2 infusion for 7 d did not significantly alter body composition of wild-type or *Ghrl*−/− mice (data not shown). MT-II infusion for 7 d decreased percent fat mass and free

fluids and increased percent lean mass, compared with baseline (d −7) in both wild-type and *Ghrl*−/− mice (*P* < 0.05 *vs.* d −7). We measured plasma acylated ghrelin levels in a cohort of chow-fed wild-type and *Ghrl*−/− mice (n = 10 mice/group; 5 months old, 31.8 g average body weight) and confirmed that circulating ghrelin is not detectable in *ghrelin*-null mice (data not shown). Wild-type mice had an acyl ghrelin concentration of 61.7 ± 10.9 pM in plasma. These results indicate that the effects of NOX-B11-2 on body weight loss and food intake suppression in mice are directly mediated by neutralization of ghrelin.

Discussion

Here we demonstrate that neutralization of endogenous bioactive ghrelin reduces ghrelin-induced and normal food intake and causes mechanism-based body weight loss in mice. We administered *in vivo* a novel type of ghrelin-blocking agent, the RNA SPM NOX-B11-2, which inhibited ghrelin action *in vitro*. NOX-B11-2 blocked ghrelin-mediated activation of GHS-R1a in cell culture (IC$_{50}$ ~7 nM), whereas a nonspecific sequence had no effect. NOX-B11-2 also inhibited human and mouse GHS-R1a activation in β-lactamase re-
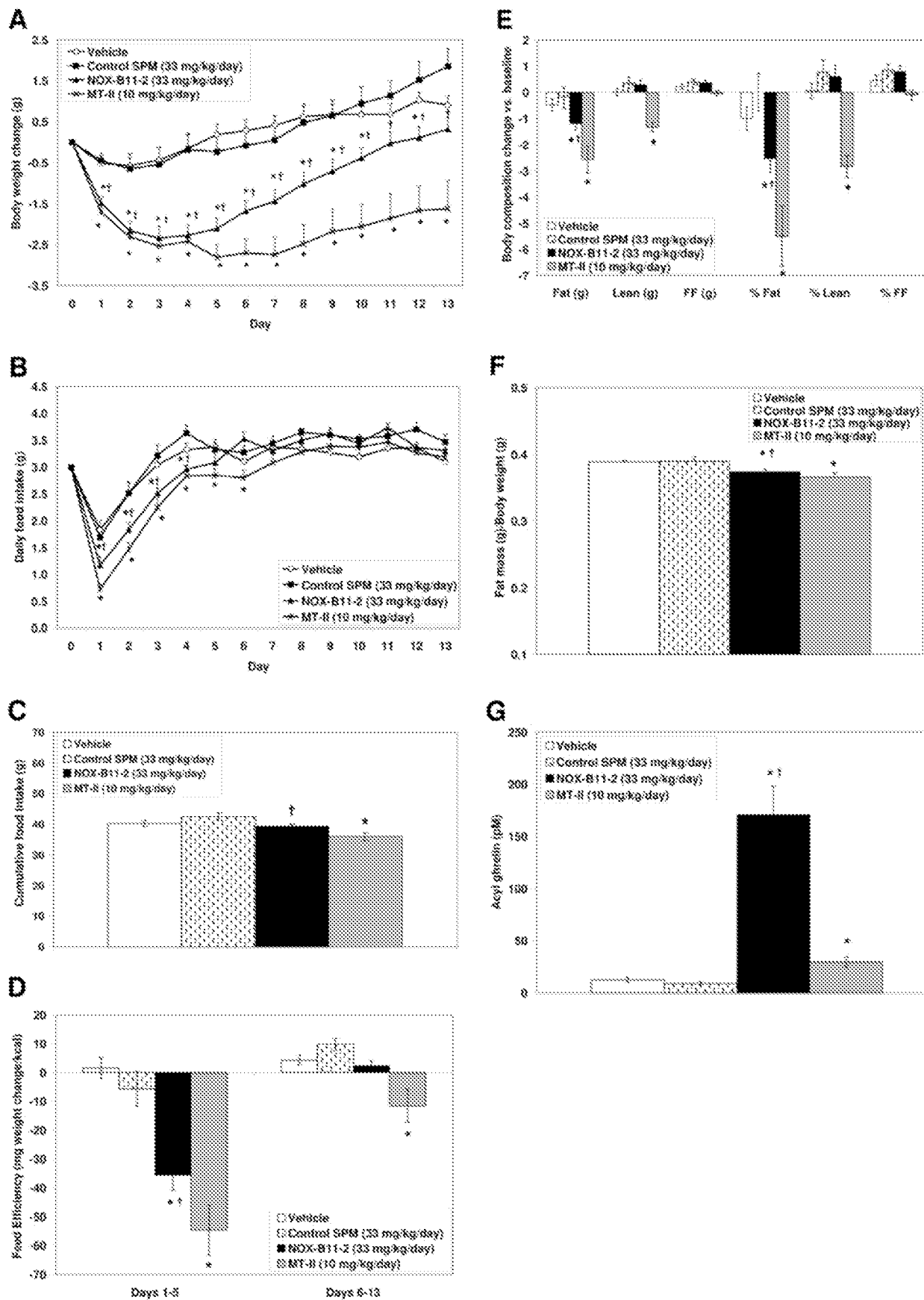
TABLE 2. Effects of chronic NOX-B11-2 infusion on plasma hormones in DIO mice

	Vehicle (PBS)	Control SPM (33 mg/kg·d)	NOX-B11-2 (33 mg/kg·d)	MT-II (10 mg/kg·d)
Leptin (ng/ml)	82.7 ± 5.6	68.8 ± 4.5	68.1 ± 2.0^a	64.9 ± 5.4^a
Insulin (ng/ml)	35.1 ± 4.1	32.9 ± 5.3	32.4 ± 6.1	23.3 ± 2.1^a
IGF-I (ng/ml)	339.7 ± 17.6	370.4 ± 21.6	370.3 ± 25.2	313.6 ± 24.4
Glucose (mg/dl)	188.7 ± 9.4	205.5 ± 6.8	189.6 ± 7.2	187.0 ± 5.3
Cholesterol (mg/dl)	196.3 ± 9.7	203.9 ± 12.0	182.2 ± 8.2	208.7 ± 10.3
Triglyceride (mg/dl)	128.3 ± 4.7	114.4 ± 11.8	110.3 ± 7.3^a	123.3 ± 9.8

Data represent means ± SEM.

[a] Statistical differences between compound-treated group and vehicle-treated group: $P \leq 0.05$ *vs.* vehicle. n = 9–10/group.

porter assays. We then explored the effects of acute NOX-B11-2 administration on ghrelin-induced feeding in mice. NOX-B11-2 (66 mg/kg, sc) blocked ghrelin-induced food intake and was without effect on feeding evoked by an orally active nonpeptide GHS-R1a agonist, demonstrating selectivity for the ghrelin peptide.

We demonstrated that selective ghrelin blockade effectively promoted fat and weight loss in DIO mice. Chronic infusion of NOX-B11-2 (33 mg/kg·d, sc) to DIO mice evoked weight loss and reduced food intake, feed efficiency, and fat mass, compared with vehicle or control SPM groups. In a 7-d study, DIO mice treated with an antighrelin SPM NOX-B11-2 showed body weight loss, compared with animals receiving a control SPM. This effect was directly mediated by SPM neutralization of ghrelin because the antighrelin SPM when administered to *ghrelin*-null mice showed no body weight loss.

Our results are in agreement with published studies of acute ghrelin blockade in adult animals using antighrelin antibodies, antisense oligonucleotides, and GHS-R1a antagonists. These include a study indicating that daily intracerebroventricular (central) administration of antighrelin antiserum for 5 d decreased both daily food intake and body weight in rats (20), supporting the hypothesis that endogenous ghrelin participates in the control of appetite. Other compelling evidence for the role of ghrelin in feeding regulation was shown with intracerebroventricular administration of polyclonal antighrelin antibodies, which robustly suppressed feeding in rats (3). Using another experimental approach to attenuate GHS-R1a expression *in vivo*, transgenic rats that express an antisense GHS-R1a mRNA under the control of a tyrosine hydroxylase promoter are reported to have lower food intake, body weight, and adipose tissue than control rats (21). Recent experiments with peripherally administered GHS-R1a antagonists, such as [D-Lys-3]GH-releasing peptide-6, indicated decreased feeding in mice (22). In addition, repeated administration of [D-Lys-3]GH-releasing peptide-6 decreased body weight gain and improved glycemic control in *ob/ob* mice (22).

In contrast, deletions of the genes encoding ghrelin or its cognate receptor yield only subtle results, perhaps due to the effects of compensatory pathways during development (7, 8, 23). Whereas it was reported by one group that enhanced fat catabolism and leanness are seen when *ghrelin*-null mice are fed a high-fat diet (8), another group's *ghrelin*-null mice were phenotypically normal (7). This begs the question of whether selective ghrelin blockade in adult animals can indeed promote weight loss. We demonstrate that blockade of endogenous ghrelin, using SPMs to neutralize acylated ghrelin, evokes food intake suppression and weight loss in DIO mice. Based on this finding and a collection of supporting *in vivo* rodent data, ghrelin appears to be a key participant in energy homeostasis.

Ghrelin is thought to play a role in mealtime hunger and meal initiation. The oxyntic cells of the stomach are the primary source of circulating ghrelin (24), whose levels rise before meals and rapidly decline on feeding (6, 11). Ghrelin also responds to longer-term changes in metabolic state. Plasma ghrelin levels are elevated after food deprivation and with chronic weight loss (13). The mechanisms that underlie these fluctuations in plasma ghrelin levels are not completely understood. The gastric vagal afferent is believed to be the major pathway conveying ghrelin's signals for starvation and GH secretion from the stomach to the brain. The orexigenic effect of ghrelin is reported to be ineffective in vagotomized rats (25), suggesting that ghrelin's direct effect on the brain may be of intrinsic origin, although this is controversial. Ghrelin has been shown to be present in several regions

FIG. 4. Effect of chronic antighrelin SPM NOX-B11-2 infusion on body weight change, food intake, and body composition of DIO mice. A, NOX-B11-2 invoked weight loss, compared with controls. Significant body weight loss was observed with NOX-B11-2 infusion on d 1–10 and d 12, compared with vehicle-treated mice (*, $P < 0.05$ *vs.* vehicle), and d 1–13, compared with the control SPM infused group (†, $P < 0.05$ *vs.* control SPM). The control SPM did not alter body weight gain relative to the vehicle control. MT-II reduced body weight gain from d 1 onward (*, $P < 0.05$ *vs.* vehicle). B, NOX-B11-2 infusion reduced daily food intake of DIO mice from d 1 to 4 (*, $P < 0.05$ *vs.* vehicle; †, $P < 0.05$ *vs.* control SPM). MT-II infusion decreased daily food intake from d 1 to 6 (*, $P < 0.05$ *vs.* vehicle). Control SPM did not alter daily food intake. C, NOX-B11-2 reduced cumulative food intake from d 1 to 8, compared with the vehicle group (*, $P < 0.05$ *vs.* vehicle) and d 1 to 13, compared with the control SPM group (†, $P < 0.05$ *vs.* control SPM). Control SPM infusion did not alter cumulative food intake. MT-II reduced cumulative food intake significantly from d 1 to 13 (*, $P < 0.05$ *vs.* vehicle). D, Feed efficiency was reduced by NOX-B11-2 infusion on d 1–5, and this effect was not observed from d 6 to 13 (*, $P < 0.05$ *vs.* vehicle; †, $P < 0.05$ *vs.* control SPM). MT-II reduced feed efficiency from d 1 to 13 (*, $P < 0.05$ *vs.* vehicle). E, NOX-B11-2 infusion reduced fat mass content, compared with baseline (day −7) (*, $P = 0.05$ *vs.* vehicle; †, $P = 0.06$ *vs.* control SPM). NOX-B11-2 infusion did not affect lean mass content. Control SPM did not alter body composition. MT-II infused mice gained less fat and lean mass over the 14-d infusion period (*, $P < 0.05$ *vs.* vehicle). F, Fat mass corrected for body weight on d 13. NOX-B11-2 infusion reduced fat mass relative to vehicle and control SPM groups (*, $P < 0.05$ *vs.* vehicle; †, $P < 0.05$ *vs.* control SPM). MT-II reduced fat mass (*, $P < 0.05$ *vs.* vehicle). G, Acyl ghrelin levels were increased by NOX-B11-2 infusion relative to vehicle and control SPM groups (*, $P < 0.05$ *vs.* vehicle; †, $P < 0.05$ *vs.* control SPM). MT-II infusion increased acyl ghrelin levels (*, $P < 0.05$ *vs.* vehicle).
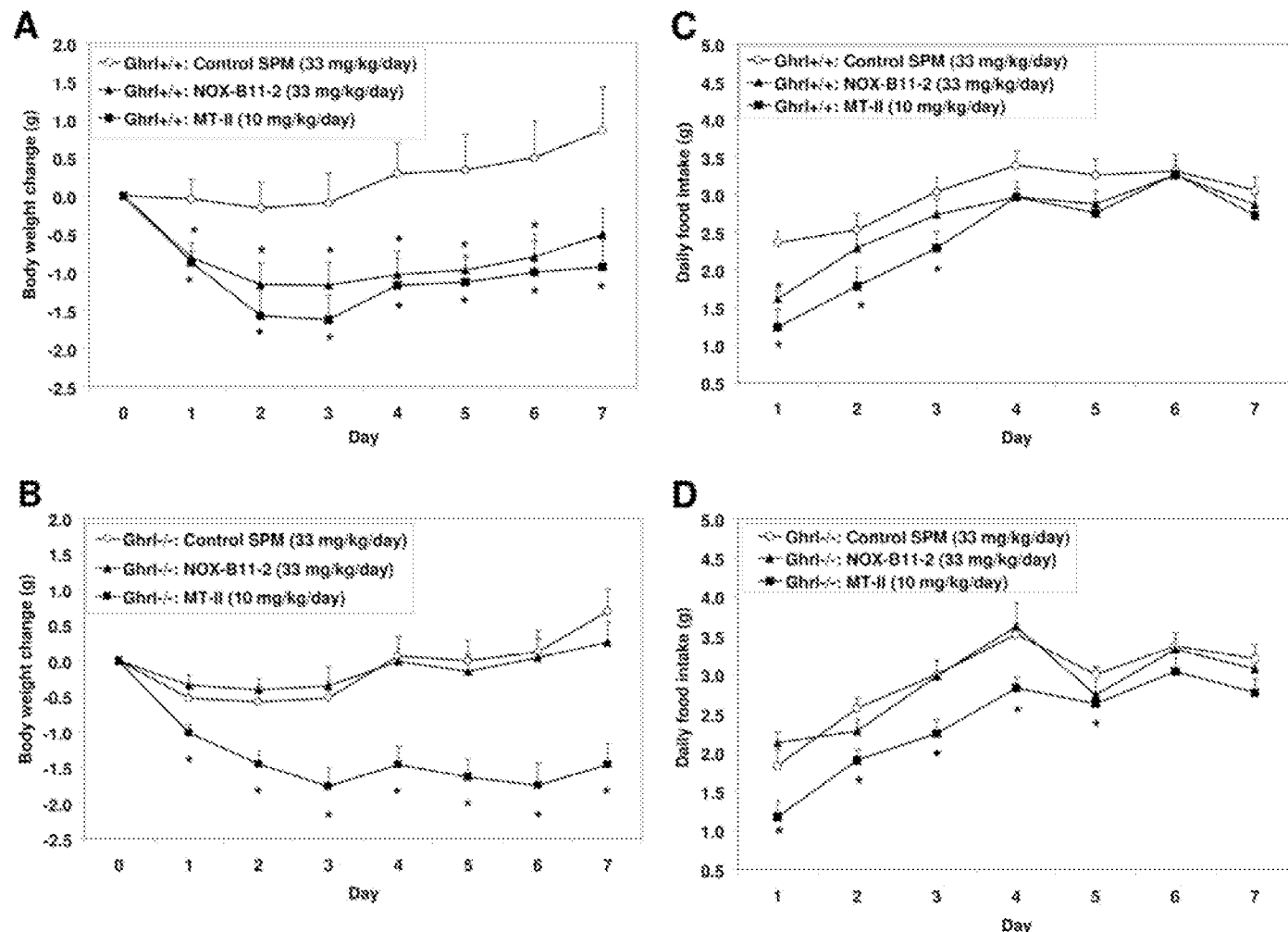
Fig. 5. Effect of chronic antighrelin SPM NOX-B11-2 infusion on food intake and body weight gain in ghrelin-deficient ($Ghrl^{-/-}$) and wild-type ($Ghrl^{+/+}$) mice. A, In wild-type mice, significant body weight loss was observed with NOX-B11-2 infusion on d 1–6, compared with wild-type mice infused with the inactive control SPM (*, $P < 0.05$ vs. control SPM). B, NOX-B11-2 did not alter body weight change of $Ghrl^{-/-}$ mice. MT-II reduced body weight gain from d 1 onward in both wild-type and $Ghrl^{-/-}$ mice, compared with their respective control SPM groups (*, $P < 0.05$ vs. control SPM). C, NOX-B11-2 infusion reduced daily food intake on d 1 in wild-type mice (*, $P < 0.05$ vs. control SPM). D, NOX-B11-2 infusion did not alter food intake of $Ghrl^{-/-}$ mice. MT-II suppressed daily food intake of wild-type mice from d 1 to 3 and $Ghrl^{-/-}$ mice from d 1 to 5 (*, $P < 0.05$ vs. control SPM).

of the hypothalamus through the use of immunohistochemical methods (2, 26–28). Hypothalamic ghrelin mRNA has been detected with the use of RT-PCR but not by in situ hybridization techniques, indicating that it is present in low abundance (2, 26–28). Neutralization of ghrelin with NOX-B11-2 infusion probably occurs in the periphery because we do not believe that the SPM is brain penetrant (i.e. SPM not detectable in brain) (15).

Tachyphylaxis is commonly observed when evaluating effects of anorectic agents (e.g. MT-II, fenfluramine) on feeding in rodent models. Possible explanations for equalization of food intake at 4–6 d in MT-II and NOX-B11-2-infused mice are receptor desensitization (changes in expression of melanocortin, ghrelin receptors) or a compensatory up-regulation of NPY and AgRP mRNA levels (29). A recent paper (30) suggests that the food ingestive pathway of the GHS-R1a is not susceptible to desensitization as transgenic mice that overexpress the human ghrelin gene and exhibit hyper-

ghrelinemia are still sensitive to the food stimulatory effects of exogenous ghrelin. In contrast, the GH secretory response is blunted in ghrelin transgenic mice with chronic hyperghrelinemia (30). These data point to a possible compensatory up-regulation in NPY and AgRP mRNA levels in animals infused with NOX-B11-2. We calculated feed efficiency (weight gain per kilocalorie ingested) to determine how efficiently the food energy was used for accretion of body mass. Feed efficiency was reduced by NOX-B11-2 infusion on d 1–5, and this effect was not observed from d 6 to 13, suggesting that the transient reduction in weight gain was not simply due to reductions in food intake. Additional factors such as enhanced thermogenesis or increased energy expenditure may play a role. Ghrelin has been shown to decrease spontaneous locomotor activity in rats (31). We did not observe any overt effects of NOX-B11-2 on locomotor activity; however, it was not measured, and thus, it is possible that ghrelin neutralization could have increased locomotor ac-

tivity of the animals. The absence of a change in feed efficiency in NOX-B11-2-infused mice from d 6 to 13 suggests that counterregulatory mechanisms were in effect to normalize body weight. These mechanisms may include counterregulatory changes in hypothalamic NPY or AgRP. In contrast, MT-II reduced feed efficiency from d 1 to 13, suggesting increased energy expenditure, consistent with previous studies (32). We did not measure energy expenditure using a calorimeter, but the changes in feed conversion efficiency with NOX-B11-2 and MT-II treatment provide indirect evidence for changes in energy expenditure.

The increases in acyl ghrelin levels in response to NOX-B11-2 infusion likely represent circulating SPM-bound ghrelin because plasma levels of SPM were more than 10-fold higher than in controls. Thus, although detectable in the ghrelin assay system, the measured ghrelin is unable to bind to the GHS-R1a. The rise of plasma ghrelin levels under NOX-B11-2 treatment can be explained by a compensatory increase in ghrelin synthesis, a prolonged lifetime of the peptide when bound to the SPM, or a combination of both. Alternatively, a portion of the increase in acyl ghrelin could represent a compensatory mechanism triggered by body weight loss, as is observed after chronic MT-II infusion albeit to a greater extent. The compensatory rise in newly synthesized ghrelin could result in the observed food intake normalization, alteration in feed efficiency (and presumably energy metabolism), and rebound body weight gain from d 6 to 13. All SPMs are cleared renally. Their half-lives in circulation vary but generally are between 10 and 14 h for a PEG-modified SPM. After iv and ip injection of NOX-B11-2 in mice, we observed a half-life around 8 h. With sc injections, we observed a longer half-life of 12 h due to the much longer time to reach maximum concentration (Helmling, S., and S. Klussmann, unpublished data).

The continuous infusion paradigm with NOX-B11-2 led to a large magnitude body weight loss for 5 d followed by rebound body weight gain from d 6 to 13 of infusion. The magnitude of the body weight loss appeared to wane during the course of the study. Continuous sequestration of acyl ghrelin and prevention of its degradation might have initiated a cascade of events that led to eventual compensation and rebound body weight gain after 5–7 d of infusion. Because the effects on weight appeared to be biphasic, longer follow-up with infusion may show that long-term weight loss is not achieved. We expect that counterregulatory effects might not be as evident with an intermittent dosing paradigm. Dosage pattern (*i.e.* intermittent bolus injection *vs.* continuous infusion) is likely to yield different pharmacokinetics of the SPM and thus potentially different effects on food intake and body weight loss. For example, intermittent iv infusions of peptide YY (3–36) produce a sustained reduction in daily food intake (33), whereas chronic sc infusion by osmotic minipump produces a transient 3- to 4-d reduction in daily food intake in rodents (34). Indeed, a multiple-day dosing study using an intermittent injection dosing paradigm may be a worthwhile approach to study long-term effects of antighrelin SPM treatment.

In summary, we presented evidence that neutralization of acylated ghrelin reduces ghrelin-induced and normal food intake and causes mechanism-based body weight loss in mice. These findings indicate a critical role for ghrelin in body weight regulation and support the notion that ghrelin is a unique anabolic counterpart to leptin and insulin in energy homeostasis. Our data suggest that NOX-B11-2 could be an innovative approach to inhibit biological action of circulating ghrelin. The decreased obesity observed in SPM-treated DIO mice provides validation for ghrelin neutralization as a potential antiobesity therapy. NOX-B11-2 could be an effective antiobesity therapy or have utility in the treatment of conditions associated with hyperphagia and elevated plasma ghrelin levels, such as Prader-Willi syndrome (14).

References

1. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JA, Smith RG, Van der Ploeg LHT 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974–977
2. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
3. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198
4. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DGA, Ghatei MA, Bloom SR 2000 The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325–4328
5. Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR 2001 Ghrelin causes hyperphagia and obesity in rats. Diabetes 50:2540–2547
6. Tschöp M, Smiley DL, Heiman ML 2000 Ghrelin induces adiposity in rodents. Nature 407:908–913
7. Sun Y, Wang P, Zheng H, Smith RG 2004 Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci USA 101:4679–4684
8. Wortley KE, Anderson K, Garcia K, Murray JD, Malinova L, Liu R, Moncrieffe M, Thabet K, Cox HJ, Yancopoulos GD, Wiegand SJ, Sleeman MW 2004 Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proc Natl Acad Sci USA 101:8227–8232
9. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, Shen Z, Marsh DJ, Feighner SD, Guan XM, Ye Z, Nargund RP, Smith RG, Van der Ploeg LHT, Howard AD, MacNeil DJ, Qian S 2004 Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. Endocrinology 145:2607–2612
10. Toshinai K, Date Y, Mrakami N, Shimada M, Modal MS, Shimbara T, Guan J-L, Wang Q-P, Funahashi H, Sakurai T, Shioda S, Matsukura S, Kangawa K, Nakazato M 2003 Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506–1512
11. Tschöp M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C 2001 Postprandial decrease of circulating human ghrelin levels. J Endocrinol Invest 24:RC19–RC21
12. Wren AM, Seal LJ, Cohen MA, Byrnes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR 2001 Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 86:5992–5995
13. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, Purnell JQ 2002 Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 346:1623–1630
14. Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS,

Schwartz MW, Basdevant A, Weigle DS 2002 Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med 8:643–644

15. Helmling S, Maasch C, Eulberg D, Buchner K, Schröder W, Lange C, Vonhoff S, Wlotzka B, Tschop MH, Rosewicz S, Klussmann S 2004 Inhibition of ghrelin action *in vitro* and *in vivo* by an RNA-SPM. Proc Natl Acad Sci USA 101:13174–13179

16. Kobelt P, Helmling S, Stengel A, Wlotzka B, Andresen V, Klapp BF, Wiedenmann B, Klussmann S, Monnikes H 30 June 2005 Anti-ghrelin SPIEGELMER NOX-B11 inhibits neurostimulatory and orexigenic effects of peripheral ghrelin in rats. Gut 10.1136/gut2004.061010

17. Nargund R, Ye Z, Tata J, Lu Z, Hong Q, Bakshi R, Mosley R, Feighner S, Pong S, Cheng K, Schleim K, Jacks T, Hickey G, Tamvakopoulos C, Cowell L, Howard A, Smith R, Patchett A, L-166446, a second generation growth hormone secretagogue. Proc 222nd American Chemical Society Meeting, Chicago, IL, 2001 (Abstract MEDI-184)

18. Wlotzka B, Leva S, Eschgfaller B, Burmeister J, Kleinjung F, Kaduk C, Muhn P, Hess-Stumpp H, Klussmann S 2002 *In vivo* properties of an anti-CaBH SPM: an example of an oligonucleotide-based therapeutic substance class. Proc Natl Acad Sci USA 99:8898–8902

19. Hill SJ, Baker JG, Rees S 2001 Reporter-gene systems for the study of G-protein-coupled receptors. Curr Opin Pharmacol 1:526–532

20. Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M, Kangawa K 2002 Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. J Endocrinol 174:283–288

21. Shuto Y, Shibasaki T, Otagiri A, Kuriyama H, Ohata H, Tamura H, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I 2002 Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. J Clin Invest 109:1429–1436

22. Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA, Kasuga M 2003 Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. Gut 52:947–952

23. Sun Y, Ahmed S, Smith RG 2003 Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23:7973–7981

24. Date Y, Kojima M, Hosoda H, Sawaguchi A, Modal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141:4255–4261

25. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M 2002 The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 123:1120–1128

26. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661

27. Mozid AM, Tringali G, Forsling ML, Hendricks MS, Ajodha S, Edwards R, Navarra P, Grossman AB, Korbonits M 2003 Ghrelin is released from rat hypothalamic explants and stimulates corticotrophin-releasing hormone and arginine-vasopressin. Horm Metab Res 335:455–459

28. Jeffery PL, Duncan RP, Yeh AH, Jaskolski RA, Hammond DS, Herington AC, Chopin LK 2005 Expression of the ghrelin axis in the mouse: an exon 4-deleted mouse proghrelin variant encodes a novel C terminal peptide. Endocrinology 146:432–440

29. Bluher S, Ziotopoulou M, Bullen Jr JW, Moschos SJ, Ungsunan L, Kokkotou E, Maratos-Flier E, Mantzoros CS 2004 Responsiveness to peripherally administered melanocortins in lean and obese mice. Diabetes 53:82–90

30. Wei W, Qi X, Reed J, Wang H-Q, Wang G, Englander EW, Greeley Jr GH 6 October 2005 Effect of chronic hyperghrelinemia on ingestive action of ghrelin. Am J Physiol Regul Integr Comp Physiol 10.1152/ajpregu.00331.2005

31. Tang-Christensen M, Vrang N, Ortman S, Bidlingmaier M, Horvath TL, Tschop M 2004 Central administration of ghrelin and agouti-related protein (83-132) increases food intake and decreases spontaneous locomotor activity in rats. Endocrinology 145:4645–4652

32. Jonsson L, Skarphedinsson JO, Skuladottir GV, Watanobe H, Schioth HB 2002 Food conversion is transiently affected during 4-week chronic administration of melanocortin agonist and antagonist in rats. J Endocrinol 173:517–523

33. Chelikani PK, Haver AC, Reeve Jr JR, Keire DA, Reidelberger RD 6 October 2005 Daily, intermittent intravenous infusion of peptide YY (3–36) reduces daily food intake and adiposity in rats. Am J Physiol Regul Integr Comp Physiol 10.1152/ajpregu.00674.2005

34. Pittner RA, Moore CX, Bhavsar SP, Gedulin BR, Smith PA, Jodka CM, Parkes DG, Paterniti JR, Srivastava VP, Young AA 2004 Effects of PYY (3–36) in rodent models of diabetes and obesity. Int J Obes Relat Metab Disord 28:963–971

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.